

THE TIGHT EPITHELIUM OF THE MONGOLIAN GERBIL SUBCOMMISSURAL ORGAN
AS REVEALED BY FREEZE FRACTURING

by

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Summary

Examination of neonatal Mongolian gerbil subcommissural organ (SCO) by freeze fracture electron microscopy reveals that the epithelial layer (the ependyma) possesses distinctive and very complex tight junctions which connect the apical portions of adjacent ependymal cells. The tight junction network of the Mongolian gerbil is more extensive neonatally than in adult, and in general, more conspicuous than in other animal species. The consistent finding of such very complex tight junctions at the interface between the C.S.F. and the SCO suggests that the SCO ependyma at least in neonatal Mongolian gerbils might function as a tight epithelium with an ability to maintain steep solute gradients and high potential differences. The sealing off of the SCO towards the C.S.F. (the C.S.F.-SCO barrier) and the effective blood-brain barrier in the organ (the blood-SCO-barrier) might well be of functional significance and thus contribute to further explorations of the obscure histophysiology of the SCO. The double barrier-system is considered a possible reference area for mechanisms involved in control and/or detection of the stability of the internal environment of the brain.

Introduction

The subcommissural organ (SCO) is a specialized area of the ependyma attached to the ventral surface of the posterior commissure. It forms the lining of the roof of the cerebral aqueduct at its junction with the third ventricle and gives rise to the so-called Reissner's fiber (RF).

The functional significance of the SCO-RF complex is still a matter of speculation, although an impressive number of different hypotheses has been proposed and tested during the last century. The search goes on and new proposals from the last decade include (1) that the Reissner's fiber is involved in morphogenetic processes by promoting the normal straight growth of the spinal cord and vertebral column (Rühle, 1971), (2) that it is the adequate stimulus for the liquor contacting neurons in the central canal (Vigh et al., 1970), (3) that it may bind selectively some biogenic amines and their metabolites within the cerebrospinal fluid (CSF) (Hess and Sterba, 1973) and thus contribute to the regulation of its composition (Diederer, 1975, also see Olsson, 1958).

One of the most persistent theories postulates that the mammalian SCO is involved in the control of thirst, water balance and/or osmoregulation (Palkovits, 1965; Diederer, 1975). This was initially proposed by Gilbert (1956), who reported that destruction of the organ in rats resulted in permanent adipsia, and that the stainability of the SCO varied with changes in hydrated-dehydrated states of the rat (Gilbert, 1958). Many investigators in the field have confirmed and extended these findings, but other studies have failed to detect any antidiuretic substance in the SCO and any change

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in its activity in relation to osmotic variations (for references, see Palkovits, 1965; Sterba, 1969; Diederer, 1975). Unfortunately, many of these experimental studies were performed in rats, in which the SCO exhibits a very low secretory activity due to an inhibitory serotonergic innervation (Møllgård et al., 1978).

The junctional complex of the SCO has not previously been studied with the freeze-fracture technique which has the advantage over ultrathin sectioning of both increasing the area of examinable membrane and of providing unambiguous criteria for the identification of tight junctions (zonulae occludentes). Furthermore, the freeze-fracture appearance of the tight junction network of a given epithelium has been correlated with its function; thus the so-called tight epithelia which are able to maintain steep ionic and osmotic gradients exhibit extensive and complex tight junction networks (see Discussion).

In the present study we have examined freeze-fracture replicas of the SCO of the Mongolian gerbil, a species which has the ability to conserve water under conditions of severe water deprivation. We have chosen to work mainly with the SCO of neonatal gerbils as they demonstrate a very high secretory activity in contrast to adult gerbils (Wiklund et al., 1977). At all ages investigated the ependymal layer of the SCO exhibited complex and well-formed apical tight junctions. The presence and nature of these junctions indicate the existence of a significant CSF-SCO barrier which may well prevent an extracellular movement of ions, water and small hydrophilic molecules between CSF and the extracellular fluid of the SCO.

Material and methods

Twenty-two neonatal, juvenile and adult Mongolian gerbils of both sexes were used in the study. Following decapitation the brains were immediately removed and processed for either light or electron microscopy.

For light microscopy the brains were immersed in Bouin's fixative for 72 h and embedded in paraffin. Serial sections of 3 μm were cut strictly in the sagittal plane through the entire organ. The sections were stained routinely with toluidine blue (Kramer and Windrum, 1955) or Bock's chromalum gallocyanin method (Bock, 1966).

For electron microscopy the brains were immersed for 6 h in a fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer.

Thin section electron microscopy: Tissue blocks containing the subcommissural organ were isolated and postfixed for 2 h in 2% OsO_4 in 0.1 M cacodylate buffer. Specimens were block-stained in 0.5% aqueous uranyl acetate for 1 h, quickly dehydrated in increasing concentrations of ethanol and embedded in Epon. The blocks were carefully oriented during embedding so that survey sections would be strictly in the sagittal or frontal plane. One μm thick sections stained with toluidine blue were examined in the light microscope. Silver-to-gray thin sections were cut from selected areas and after staining with uranyl acetate and lead citrate examined in a Hitachi HS8 microscope.

Freeze-fracture: Small blocks of SCO were prepared after 6 h of fixation as described above. Following 2 h of infiltration with 25% buffered glycerol at room temperature the specimens

were mounted and oriented on gold discs so that a transverse cleavage perpendicular to the ventricular surface could be achieved. The tissue was then frozen in the liquid phase of partially solidified Freon 22 and stored in liquid nitrogen. Freeze-fracturing followed by carbon-platinum shadowing was performed with a Balzer's apparatus (BAF 301) equipped with an electron beam gun. Specimens were fractured at a stage temperature of -115°C and replicated with platinum-carbon without etching. The thickness of the platinum-carbon and carbon coats (approximately 2 nm and 20 nm) was controlled by a thin film monitor. Replicas were cleaned in chromic acid and chrome-sulphuric acid.

Results

In the Mongolian gerbil, as in other rodents, the SCO lies in the roof of the aqueduct immediately ventral and rostral to the posterior commissure at the junction between the midbrain and diencephalon. The SCO of the neonatal gerbil has already attained its adult form and is therefore large relative to other diencephalic and mesencephalic structures (Fig. 1A). In the sagittal plane it is about 800 μm long so that the entire organ can be punched out, oriented and mounted on the gold discs used in the freeze-fracture procedure. The organ consists of two parts: (1) A well-developed layer of high columnar epithelial cells (the SCO-ependyma) in contact apically with the C.S.F. and (2) a layer of basal or hypendymal cells which are detached from the ventricular surface. Together with glial cells and nerve cells, basal processes of SCO ependymal cells the SCO hypendymal cells form the SCO-hypendyma. In the neonatal and juvenile Mongolian gerbil the SCO-ependyma is strongly Gomori-positive (Fig. 1B).

This study is limited to the examination of intercellular junctions at the C.S.F.-SCO interface (the junctions between apical processes of SCO ependymal cells) and does not include those of the basal processes, the hypendyma and the vascular bed. In describing the freeze-fracture appearance of the junctions, the protoplasmic fracture (P face) is the fracture surface of the protoplasmic half of the membrane, whereas the exoplasmic fracture face (E face) is the fracture surface of that half of the membrane facing the extracellular space.

By thin section electron microscopy the SCO ependymal cells are seen to be linked at their apical (ventricular) surface by

an extensive and complicated junctional complex (Fig. 2), which extends for about 3 μm down the lateral surfaces. Multiple, punctate close appositions of adjacent cell membranes are found throughout the apical two thirds of every junctional complex. The membrane appositions have an overall width of approximately 14nm measured between the cytoplasmic face of the constituent membranes and exhibit a pentalaminar appearance in uranyl block-stained material. They are therefore considered to belong to a tight junction network.

A characteristic zonula adherens with a typical dense filamentous network on the cytoplasmic surface forms the basal component of the junctional complex. The space within this junction which sometimes contains a midplane density remain at a fairly constant width of about 20nm. Characteristic desmosomes have not been identified in the junctional complex and due to the small distance between the focal membrane fusions of the tight junction network the existence of gap junctions cannot be excluded by thin section electron microscopy. Except for a smaller junctional depth (cf. below) we found no differences between apical junctional complexes in subcommissural organ of neonatal, juvenile and adult Mongolian gerbils.

Freeze fracturing: The junctional region of SCO ependymal cells is well defined because the latter exhibit a highly characteristic apical surface with abundant microvilli and very few cilia which protrude into the CSF from a dome-shaped apical membrane. The tight junction network seems to form a complete belt-like structure around each ependymal cell, since it is present whenever the apical part of the lateral cell membrane is exposed during the fracture procedure.

The tight junction network in the SCO of neonatal Mongolian gerbils (Figs. 3, 4) is more complex than the network found in adult animals (Figs. 5A, B).

In the neonatal period all investigated tight junction networks had at least 10 complete strands, and many showed 25-30 (Fig. 3B). The network is highly cross-linked with a preferential orientation of the strands parallel or perpendicular to the cell surface. The junctional depth (the distance between apical and basal strands) varies between 1 and 3 μm . Neither "complex" strands (cf. Møllgård et al., 1979) nor gap junctions were observed within or as a part of the network.

In adult gerbils the tight junction network was characterized by 5-15 strands arranged in an interconnected, apical and basal network. The portion nearest to the CSF exhibited 3-6 strands oriented parallel to the cell surface and with a regular interstrand spacing, whereas the more basal strands formed an elongated, loose network (Figs. 5 A and B). Gap junctions were not observed as a part of the network.

Discussion

The main result presented in this freeze fracture study is that the ependymal cells of the SCO of the Mongolian gerbil are joined by very complex tight junctions. The fact that the SCO is sealed off from the CSF might well be important for an understanding of the obscure histophysiology of the organ. Conflicting data with regard to this question appeared simultaneously some years ago from two different groups both working with the adult rabbit SCO. Weindl and Joynt (1973) reported that horseradish peroxidase (HRP) after intraventricular injection enters the intercellular spaces of the SCO through apical gap junctions, and they found no tight junctions in this location. An investigation from our laboratory (Kimble et al., 1973) based on ventriculo-cisternal perfusion of ruthenium red demonstrated, however, the existence of typical apical tight junctions. Freeze fracture investigations of the SCO of fetal, neonatal and adult rabbits and rats suggest the existence of simple, but complete tight junctions at the CSF-SCO interface also in these species (our unpublished data). These findings and the results of the present study thus confirm and extend our previous observations. The reason for the conspicuous variations between animal species is unknown.

The SCO is included in the group of specialized areas of ependyma, which are collectively called circumventricular organs. In contrast to the usual ependymal lining of the ventricular system the ependyma overlying these organs is generally held to be more or less impermeable to CSF-borne dyes and proteins due to the presence of tight junctions between adjacent ependymal cells (for a review see Brightman et al., 1975). The SCO thus seems to conform to the general description of the circumventricular organs

with respect to the sealing off of the ependyma. The tight junctions in areas with fenestrated leaky capillaries presumably serve to maintain the insulation of the bulk of the C.N.S. from unrestricted access of material from the blood, but the degree of tightness of the median eminence ependymal tight junctions is probably even less than that of choroid plexus epithelial cell tight junctions (Brightman et al., 1975).

Although it is commonly held that the SCO like other circumventricular organs has no blood-brain barrier (see eg. Löfgren, 1965 and review by Koella and Sutin, 1967) this does not appear to be the case. Thus, Wislocki and Leduc (1952 a,b) did not include the subcommissural organ in their list of areas outside the blood-brain barrier after an extensive study using trypan blue and silver nitrate, and a whole range of vital stains which colored other circumventricular organs were never observed inside the SCO in either neonatal or adult rats, rabbits and gerbils (our unpublished data). Furthermore, intravenously injected HRP did not penetrate into the organ in rabbits (Weindl and Joynt, 1973), rats (von Bomhardt et al., 1974) and mice (Broadwell and Brightman, 1976) indicating that the capillaries of the SCO do not differ from typical brain capillaries with respect to the effectiveness of the blood-brain barrier.

The suggested classification of epithelia as "tight" or "leaky" is based on the measured transepithelial resistance of these systems (Frömter and Diamond, 1972). The principle variation in conductance appears to be the degree of paracellular permeability rather than the permeability of cell membranes which are the principle resistive elements of the transcellular

route for ion movement. Paracellular permeability, apparently not related to the active transport of ions, is largely limited by the tight junctions (Farquhar and Palade, 1963) which characteristically occur at the luminal end of the epithelial intercellular space. Claude and Goodenough (1973) have attempted to correlate the physiological "tightness" of the junctions of a given epithelium with the number of sealing strands comprising the networks. So-called "leaky" junctions, such as those found in the proximal convoluted tubule of the mammalian kidney, have junctions composed of only one or two strands, while the physiologically "tight" junctions, such as those which seal the amphibian urinary bladder, have 4-11 strands and are more than $0.5\ \mu\text{m}$ in depth. More recently, two studies have suggested that the above-mentioned correlation may not be an absolute one (Martinez-Palomo and Erlij, 1975; Møllgård et al., 1976). Available data indicate, however, that epithelia with a very high strand number (e.g. more than 15) do belong to the group of physiological tight epithelia.

No measurements of transepithelial resistance are available for the SCO ependyma of any species, but the findings in neonatal Mongolian gerbils of up to 30 strands in SCO ependymal junctions in a broad band ($1-3\ \mu\text{m}$ wide) would still suggest that this epithelium is of a very tight variety. In contrast, the strand number in adults is within the range (4-10) in which no definite statement on tightness would appear to be justified. At present we have no explanation for this marked developmental difference in tight junction morphology, but some specific mechanisms for control and/or detection of the ionic composition of the internal

brain environment may conceivably operate in the neonatal period of a desert animal.

Another implication of the tight C.S.F.-SCO barrier is that secretory material released from SCO-hypendymal cells would not be expected to penetrate from the extracellular space of the SCO out into the C.S.F. A secretory product is more likely to be destined for the perivascular space which is in direct communication with the extracellular space of the organ. Thus, the present results strongly support the idea of secretion towards the blood vessels - the so-called basal secretion (cf. Oksche, 1969; Kimble and Møllgård, 1975).

The presence of a C.S.F.-SCO barrier and the effective blood-brain barrier in the organ might well be of functional significance and thus contribute to further explorations of the obscure histophysiology of the SCO. The significant double barrier system of the SCO of neonatal Mongolian gerbils would be well suited as a reference area for mechanisms involved in control and/or detection of the stability of the internal environment of the brain.

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Legends

Figs. 1A & B: Adjacent sagittal Gomori-stained sections of the meso-diencephalic region of a neonatal Mongolian gerbil. The rectangle in Fig. 1A encloses the SCO. Left is anterior. The cerebral aqueduct is labelled by asterisks and the pineal stalk (triangle) is seen above the organ. Bar indicates 1 mm. A higher magnification is shown in Fig. 1B. Note the strong Gomori-positive reaction in the ependymal layer of the organ (arrows). Bar indicates 100 μ m.

Fig. 2. Electron micrograph of the apical junctional complex between adjacent SCO ependymal cells in the neonatal gerbil. Note the multiple punctate close appositions (arrows). In the deepest region of the junction the apposed membranes are separated by 20 nm and lined with a thin coat of amorphous dense material. The extracellular space is narrowed at intervals (triangles) presumably corresponding to basal strands in the tight junction network. Bar indicates 0.1 μ m.

Figs. 3A & B: Extensive and highly cross-linked tight junction networks between adjacent SCO-ependymal cells in neonatal gerbil. Apart from the most luminal strands which are mainly oriented parallel to the cell surface (3A), the long strands in the network are preferentially arranged perpendicular to the cell surface (3B). Note the absence of gap junctions. Asterisks indicate luminal cell membranes. Bars indicate 0.5 μ m.

Figs. 4, 5A & 5B: Compare the junctional depth, strand number and geometrical pattern of the tight junction network from neonatal (4) and adult gerbil SCO (5A & B). The junctional depth is indicated by arrow heads. In the adult stage the 3-6 most apical strands (grooves on the E face in 5A, and ridges on the P face in 5B) are oriented mainly parallel to the cell surface, and the interstrand spacing appears regular. The more basal strands form an elongated, loosely interconnected network. The micrographs are at the same magnification, and the bar indicates 0.5 μ m.







